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Rice Os4BGlu12 is a wound-induced β-glucosidase that hydrolyzes cell wall-β-glucan-derived oligosaccharides and glycosides

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ABSTRACT

Rice Os4BGlu12 β-glucosidase is a family 1 glycoside hydrolase, the transcript levels of which have previously been found to be induced in response to herbivore attack and salinity stress. Here, high levels of *Os4bglu12* transcripts were also detected in the shoot during germination, in the leaf sheath and stem of mature rice plants under normal growth conditions. The transcripts of this gene were up-regulated in response to wounding, methyl jasmonate and ethephon in 10-day-old rice seedlings. Os4BGlu12 expressed in recombinant *Escherichia coli* hydrolyzed β-(1,3;1,4)-glucooligosaccharides generated by the wounding-induced rice endo-(1,3;1,4)-β-glucanase OsEGL1, suggesting that both enzymes may act in concert in remodeling of damaged cell wall. Among oligosaccharides tested, Os4BGlu12 hydrolyzed β-(1,4)-linked glucooligosaccharides with highest catalytic efficiency (k_{cat}/K_m = 2.7–4.9 s^{-1} mM^{-1}) when the degree of polymerization ranged from 3 to 6. It also hydrolyzed the β-(1,3)-linked disaccharide laminaribiose with high catalytic efficiency (k_{cat}/K_m = 4.5 s^{-1} mM^{-1}). Among the natural glycosides tested, Os4BGlu12 efficiently hydrolyzed deoxycorticosterone 21-glucoside (k_{cat}/K_m = 20 s^{-1} mM^{-1}) and apigenin 7-*O*-β-D-glucoside (k_{cat}/K_m = 6.7 s^{-1} mM^{-1}). The amino acid residues predicted to line the active site of Os4BGlu12 are more similar to those of cyanogenic and flavonoid β-glucosidases than oligosaccharide hydrolases, and it may function in defense, as well as in cell wall-derived oligosaccharide break-down.

1. Introduction

Plant glycosyl hydrolase family 1 (GH1) β-glucosidases (EC 3.2.1.21) hydrolyze the β-*O*-glycosidic bond at the anomeric carbon of glucose moieties at the nonreducing end of carbohydrate or glycoside molecules [1]. The glycones recognized by GH1 β-glycosidases include glucose, galactose, fucose, mannose, xylose, 6-phospho-glucose and 6-phospho-galactose. The diversity of aglycones is higher, including monosaccharides, oligosaccharides and aryl or alkyl groups. The physiological functions of these enzymes in plants based on the activities of the aglycone moieties of substrate include (1) defense against pathogens and herbivores [2–4], (2) phytohormone activation [5,6], (3) lignification [7], (4) cell wall catabolism [8,9] and (5) release of active metabolic intermediate molecules [10]. The GH1 enzymes may hydrolyze substrates with a broad range of different glycones or aglycones with different specificities, but some enzymes may be specific for only one type of glycone or aglycone. The fundamental substrate specificity of these enzymes depends on the overall dimensions and geometry of the binding site and the distribution of the active site amino acids that are important for the substrate recognition and binding, which complement the structure of the aglycone and glycone moieties of the substrate [11,12].

Forty genes homologous to GH1 β-glucosidases have been identified in rice genomic sequences, 34 of which appear to be functional in rice [13]. To date, only a few rice β-glucosidase isoenzymes have been characterized for their possible function. Partially purified β-glucosidases from rice were described that

Abbreviations: DP, degree of polymerization; ESI-MS, electrospray ionization-mass spectroscopy; GH1, glycosyl hydrolase family 1; GLC, gas liquid chromatography; GST-OsEGL1, glutathione-*S*-transferase-rice endoglucanase OsEGL1 fusion protein; MS, mass spectroscopy; PMAAs, partially methylated alditol acetates; *p*NP, *p*-nitrophenyl; TLC, thin layer chromatography; Trx-Os4BGlu12, thioredoxin-rice β-glucosidase Os4BGlu12 fusion protein.

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hydrolyze gibberellin glucosides [5]. Akiyama et al. [9] determined the N-terminal sequence of a cell wall-bound β-glucosidase that preferentially hydrolyzed cellooligosaccharides and laminarioligosaccharides. Opassiri et al. [14] reported the recombinant rice BGlu1, later designated Os3BGlu7, showed strong hydrolysis and glucotransferase activity with cellooligosaccharides and laminarioligosaccharides and hydrolyzed natural glycosides with low activity. Recently, Kuntothom et al. [15] characterized two rice β-glycosidases which were grouped in the same phylogenetic cluster with Os3BGlu7 β-glucosidase and plant β-mannosidases. Os3BGlu8, which has a protein sequence similar to Os3BGlu7, hydrolyzed cellooligosaccharides but not mannooligosaccharides, while Os7BGlu26, the sequence of which is more closely related to plant β-mannosidases, could hydrolyze both cellooligosaccharides and mannooligosaccharides. Seshadri et al. [16] reported that recombinant rice Os3BGlu6 preferentially hydrolyzed *n*-octyl β-D-glucoside and β-(1,3)- and β-(1,2)-linked disaccharides, and hydrolyzed apigenin 7-*O*-β-D-glucoside and several other natural glycosides, but at low catalytic rates.

Previously, the *Os4bglu12* cDNA (rice genome locus Os04g0474800) for a rice β-glucosidase, which encodes a protein with high amino acid sequence identity with the N-terminal sequence of a cell wall-bound β-glucosidase purified from rice [9], was expressed as a soluble active protein in *Escherichia coli* [13]. The preliminary analyses showed that it possessed hydrolytic activity on cellooligosaccharides and laminaribiose. While its activity appeared to be somewhat similar to that of the rice Os3BGlu7, Os3BGlu8 and Os7BGlu26 β-glucosidases, Os4BGlu12 was classified in a distinct group on the phylogenetic tree, more closely related to dicotyledon defense β-glucosidases. Functional genomics studies have shown that the transcript level of the *Os4BGlu12* gene is induced in response to herbivore attack and salinity stress [17,18], but a thorough analysis of its expression patterns has yet to be reported.

To further investigate the physiological function of rice Os4BGlu12, we determined the expression patterns of this gene in rice tissues and in rice seedlings after wounding and treatment with phytohormones by RNA gel blot analysis. Kinetic analysis of the enzyme was also performed to evaluate the substrate specificity of Os4BGlu12 against various artificial glycosides, oligosaccharides and natural glycosides.

2. Materials and methods

2.1. Materials

The soluble active recombinant thioredoxin-Os4BGlu12 fusion protein (Trx-Os4BGlu12) was expressed in Origami B(DE3) *E. coli*, and purified as described previously [13]. The rice OsEGL1 endo-(1,3;1,4)-β-glucanase was expressed as a glutathione-*S*-transferase fusion protein, GST-OsEGL1, in DH5α *E. coli* and purified as described previously [19]. Sophorose, *p*-nitrophenyl (*p*NP) glycosides, D-amygdalin, indoxyl β-D-glucoside, salicin, phlorizin dihydrate, linamarin, naringin, deoxycorticosterone 21-glucoside, and gossypin were purchased from Sigma Chemical Co. (St. Louis, MO). Apigenin 7-*O*-β-D-glucoside and quercetin 3-β-D-glucoside were purchased from Fluka (Steiheim, Swizerland). Cellooligosaccharides with degree of polymerization (DP) of 2–6 and laminarioligosaccharides (DP 2–4) were from Seikagaku Kogyo Co. (Tokyo, Japan).

2.2. RNA gel blot analysis

Rice (*Oryza sativa* L. cv. Yukihikari) seeds were germinated in the dark for 4 days at 28 °C and then grown in a 12 h light–12 h dark cycle from day 4 to day 10 at 28 °C and moistened with sterile distilled water. Some 7-day-old seedlings were harvested and dissected into separate parts (shoot, root and endosperm). Other seedlings were transferred to soil and grown for an additional 4–5 weeks to reach the flowering stage. Rice plants were harvested and separated to six parts (flower, stem, root, node, leaf blade and leaf sheath). Ten-day-old rice seedlings were treated with wounding, 10^{-4} M methyl jasmonate and 10^{-4} M ethephon for different time courses. All plant samples were kept at −70 °C for RNA isolation.

A gene-specific probe for *Os4bglu12* was amplified from a rice genomic DNA as the template with the 445-3′end forward (5′-ATGGAGCAAACGTGAAGGGAT-3′) and 445-3′UTR reverse (5′-AACTGGATTACTTCCATCTC-3′) primers derived from the coding sequence at the C-terminal part and 3′-untranslated region of the gene, respectively. The amplification was performed with 30 cycles at 94 °C 45 s, 45 °C 45 s, and 72 °C 1 min, using *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN).

Total RNA was isolated from rice tissues by the SDS–phenol method described by Bachem et al. [20]. Thirty micrograms of total RNA from each sample was denatured and electrophoresed on a 1.2% formaldehyde–agarose gel and transferred onto a Hybond N+ nylon membrane (GE Healthcare, Buckinghamshire, UK) by standard procedures [21]. The probe was labeled by Rediprime II random priming with α-[^{32}P]dCTP (GE Healthcare) and used for hybridization with RNA blots for 16 h at 42 °C. The blots were then washed once with 0.1% SDS, 2× SSC for 30 min at 65 °C and washed twice with 0.1% SDS, 0.1× SSC for 15 min at 65 °C, then exposed to a Fuji film imaging plate for 16 h at room temperature. The positions of radioactive bands were visualized with a Fuji Film BAS 1000 BioImaging Analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.3. Hydrolysis of crude cell walls by recombinant rice OsEGL1 and Os4BGlu12

Crude cell wall was prepared by grinding 10-day-old rice seedling leaves to a fine powder and then washing the powder 4 times with 2 vol. of 80 °C 90% (v/v) ethanol, and the cell wall pellets were dried under vacuum. In a 10 mL reaction containing 300 mg crude cell wall powder, 100 pg of the GST-OsEGL1 in 50 mM sodium acetate, pH 5.0, was incubated at 37 °C for 6 h. The reaction was stopped by boiling for 10 min, and then centrifuged at 7000 × *g* for 20 min. The supernatant was desalted with AG501-X8D ion exchange resin (Bio-Rad, Richmond, CA) and then evaporated to dryness under vacuum. The concentrated fraction was dissolved in 200 μL distilled water and then separated by thin layer chromatography (TLC) as described previously [22]. To isolate each sugar product, only edge parts (1 cm in width) of both sides of the developed silica gel plate were cut off for detection of sugar spots, and the silica gel layer at the positions of the sugars was scratched off from the unstained plate and packed into a column (1.5 cm inner diameter, 9 cm in length). The sugars were eluted from a column with 5 vol. of 80% (v/v) ethanol and evaporated to dryness under vacuum.

The cell wall hydrolysis products were assayed with 1 μg Trx-Os4Glu12 in 50 mM sodium acetate, pH 5.0, in 50 μL reaction volume at 37 °C for 0, 10, 30 and 60 min. The reaction was stopped by boiling for 10 min and the products were detected by TLC as described above.

2.4. Chemical characterization of cell wall hydrolysis products

Molecular weights of purified cell wall hydrolysis products were analyzed by electrospray ionization-mass spectrometry (ESI-MS). ESI-MS measurements were performed on a time-of-flight mass spectrometer JMS-T100CS (JEOL, Japan) using direct injection. The measurement conditions were as follows: scan mode, positive

Table 1
Kinetic parameters of rice Os4BGlu12 in the hydrolysis of oligosaccharides, *p*NP glycosides and natural glycosides.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Cellooligossaccharides (DP[a])			
2	1.8 ± 0.3	25 ± 3	0.073 ± 0.003
3	12 ± 2	4.6 ± 0.0	2.7 ± 0.3
4	17 ± 1	5.7 ± 0.5	3.0 ± 0.2
5	24 ± 3	4.8 ± 0.5	4.9 ± 0.1
6	18 ± 2	4.8 ± 0.6	3.8 ± 0.2
Laminarioligosaccharides (DP)			
2	23 ± 1	5.1 ± 0.2	4.5 ± 0.0
3	ND[b]	ND	ND
4	ND	ND	ND
Sophorose	5.2 ± 0.3	4.4 ± 0.4	1.2 ± 0.1
(1,3;1,4)-Glucotriose	0.84 ± 0.04	4.9 ± 0.9	0.18 ± 0.03
(1,3;1,4)-Glucotetraose	2.5 ± 0.2	3.3 ± 0.3	0.77 ± 0.08
*p*NP β-D-glucoside	20 ± 1	0.80 ± 0.08	24 ± 1
*p*NP β-D-fucoside	18 ± 1	0.39 ± 0.04	47 ± 2
*p*NP β-D-galactoside	12 ± 1	1.4 ± 0.2	8.5 ± 1.3
*p*NP β-D-xyloside	6.0 ± 0.3	0.24 ± 0.04	25 ± 0
*p*NP α-L-arabionoside	2.3 ± 0.1	0.12 ± 0.03	23 ± 4
Deoxycorticosterone 21-glucoside	13 ± 2	0.68 ± 0.18	20 ± 2
Indoxyl β-D-glucoside	2.9 ± 0.2	4.4 ± 0.5	0.67 ± 0.02
D-Amygdalin	0.25 ± 0.01	5.8 ± 0.2	0.042 ± 0.000
Linamarin	0.28 ± 0.04	9.4 ± 1.4	0.031 ± 0.000
Apigenin 7-*O*-β-D-glucoside	0.83 ± 0.02	0.12 ± 0.01	6.7 ± 1.6
Phlorizin	ND	ND	ND
Quercetin 3-β-D-glucoside	ND	ND	ND
Gossypin	ND	ND	ND
Naringin	ND	ND	ND
Salicin	ND	ND	ND

[a] Means degree of polymerization.
[b] Means not detectable.

ion; needle voltage, 2.0 kV; orifice voltage, 60 V; desolvation temperature, 80 °C; sample injection rate, 6 μL min^{-1}; solvent, 0.05% aqueous trifluoroacetic acid.

To analyze the sugar linkages in products released from cell wall by rice OsEGL1, partially methylated alditol acetates (PMAAs) were prepared from each product (1–2 mg) following the method of Kim et al. [23]. The PMAAs were separated and analyzed with a JMS-AX500 (JEOL, Japan). The gas liquid chromatography (GLC) peaks were identified by mass spectrometry (MS) on an electron-impact ionization mode scanning from 50 to 500 *m/z*. PMAA standards were prepared from cellotriose and laminaritriose.

2.5. Enzyme assays and kinetic analysis

Kinetic parameters were determined for *p*NP glycosides, oligosaccharides, and commercially available natural glycosides from triplicate assays of 5–7 substrate concentrations at time points and enzyme concentrations where the reaction rate was linear and the absorbance value was in the range of 0.1–1.0. The assays were done at 37 °C in 50 mM sodium acetate, pH 5.0, which are the optimum temperature and pH for Os4BGlu12 [13]. To determine sugar specificity, in a 100 μL reaction, 1.45 pmol Trx-Os4BGlu12 was incubated with *p*NP glycosides. The reactions were stopped by adding 50 μL of 0.4 M sodium carbonate, and the amount of *p*-nitrophenol released was determined from its A_{405}. Hydrolysis of oligosaccharides and commercially available natural glycosides (listed in Table 1) was assayed in 50 μL reaction volumes containing 1.81–14.5 pmol enzyme, the reactions were stopped by boiling, and the glucose released was quantified by the peroxidase/glucose oxidase assay method, as described previously [14]. Kinetic parameters were calculated by nonlinear regression of Michaelis–Menten plots with Grafit 5.0 (Erithacus Software), based on the amount of nonreducing terminal glucosyl residues released in each reaction. The enzyme molarity was calculated based on the molecular weight of 69 kDa for Trx-Os4BGlu12. Protein assays were performed by the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Apparent subsite affinities were calculated from the k_{cat}/K_m values by the method of Hiromi et al. [24].

3. Results

3.1. Expression patterns of Os4bglu12 gene in rice tissues

RNA gel blot analysis detected *Os4bglu12* transcripts in high abundance in the shoot, and at low levels in the root and endosperm in 7-day-old rice seedlings (Fig. 1). In 6-week-old mature rice plants at flowering stage, *Os4bglu12* mRNA was highly expressed in leaf

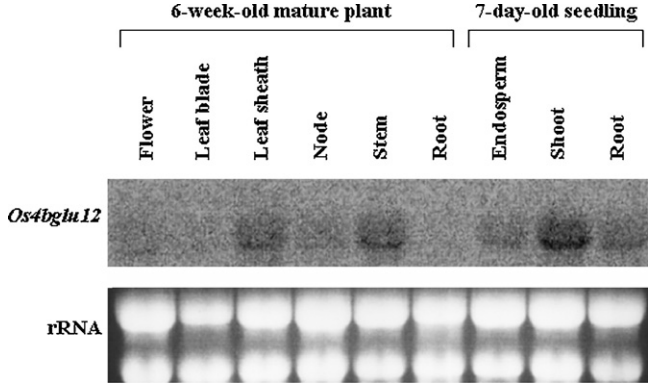

Fig. 1. RNA gel blot analysis of *Os4bglu12* expression levels in various parts of 7-day-old rice seedling and 6-week-old mature plant at flowering stage. The RNA blot was hybridized with the α-^{32}P-labeled 264 bp 3′end fragment of the *Os4bglu12* cDNA probe. Thirty micrograms of total RNA was loaded in each lane on a 1.2% agarose formaldehyde gel. The ethidium bromide-stained gel below the blot indicates the equal RNA loading before blotting.

sheaths and stems. The *Os4bglu12* transcripts were detected with moderate levels in node, flower, leaf blade and very low or no signal was seen in root.

3.2. Effects of wounding and plant hormones on expression of *Os4bglu12* gene in rice seedlings

The expression of *Os4bglu12* was induced as early as 5–10 h after wounding of 10-day-old seedlings and decreased gradually thereafter (Fig. 2A). *Os4bglu12* mRNA levels significantly increased within 10 h after treating with methyl jasmonate, after which the mRNA levels decreased slowly (Fig. 2B). Treatment with ethephon induced a rapid temporary increase in *Os4bglu12* mRNA levels within 10 h, after which the mRNA levels decreased rapidly within 1 day and then decreased gradually (Fig. 2C).

3.3. Hydrolysis of rice cell wall by rice OsEGL1 and Os4BGlu12

The wounding, methyl jasmonate and ethephon induction of expression of *Os4bglu12* gene is similar to that of rice OsEGL1 endo-(1,3;1,4)-β-glucanase, which specifically hydrolyzes (1,3;1,4)-β-glucans [19]. Os4BGlu12 was assayed with oligosaccharides released from rice cell walls by OsEGL1. As seen on the TLC, the hydrolysis of crude rice cell walls by a recombinant OsEGL1 generated three major hydrolysis products, which were denoted as Os1, Os2 and Os3 (Fig. 3). Os4BGlu12 could hydrolyze Os1, which migrated between spots of cellotetraose and cellotriose standards, and the glucose product released by the enzyme increased during incubation from 10 to 60 min (Fig. 3).

3.4. Chemical characterization of cell wall hydrolysis products

In the ESI-MS data, signals of oligosaccharides Os1, Os2, and Os3 were detected at *m*/*z* 698 ([M+Na]$^+$), 527 ([M+Na]$^+$), and 365 ([M+Na]$^+$), respectively. These data suggest Os1, Os2, and Os3 to be a tetrasaccharide, a trisaccharide, and a disaccharide, respectively. The linkage of these oligosaccharides was confirmed by methylation analysis. On the basis of their retention times in GLC and their fragmentation patterns in GC–MS, three permethylated aditol acetates of Os1 and Os2 were identified as 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol; 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Those from Os3 were identified as 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methyl-D-glucitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (data not shown). The results of the analyses confirmed

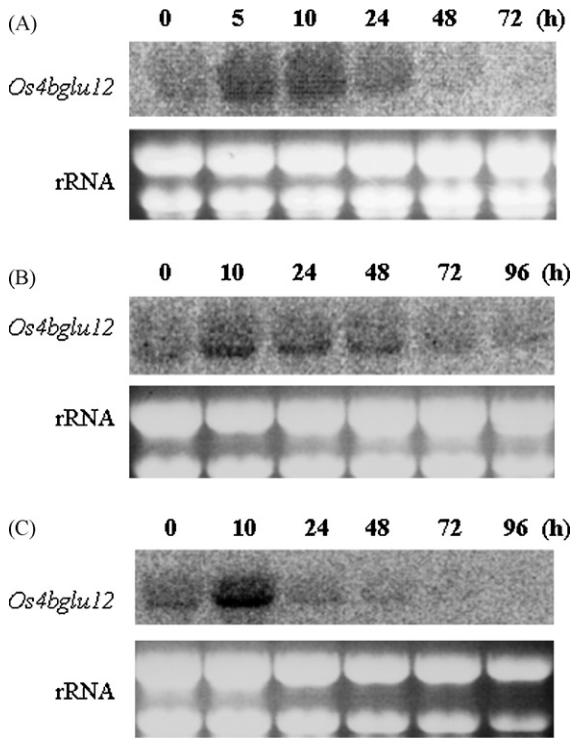

Fig. 2. RNA blot analysis of *Os4bglu12* expression in 10-day-old rice seedlings treated with (A) wounding, (B) methyl jasmonate and (C) ethephon at different time points. Thirty micrograms of total RNA was loaded on a 1.2% agarose formaldehyde gel. The RNA blot was hybridized with the α-^{32}P-labeled 264 bp 3′end fragment of the *Os4bglu12* cDNA probe. The ethidium bromide-stained gel below the blots showed the RNA loading before blotting.

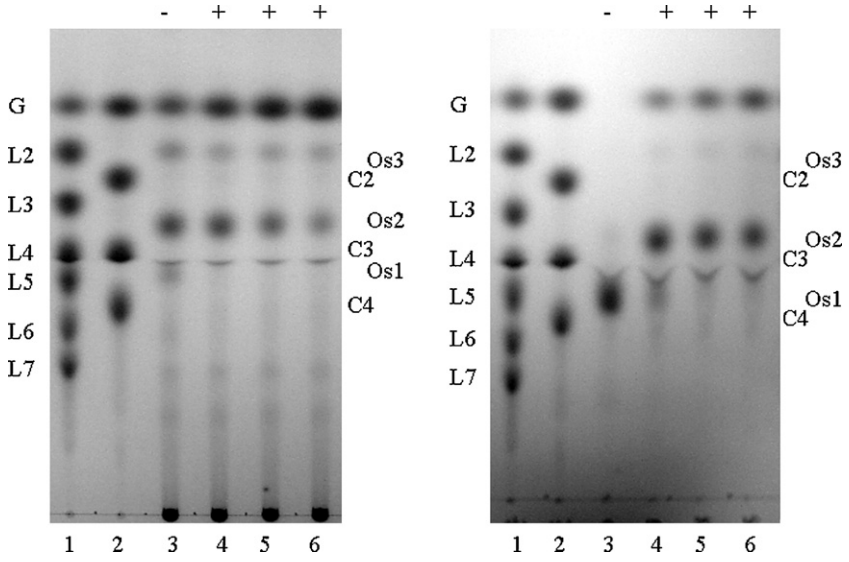

Fig. 3. TLC analysis of rice cell wall hydrolysis by rice OsEGL1 endo-(1,3;1,4)-β-glucanase and Os4BGlu12 β-glucosidase. (A) Time course of the reaction of Os4BGlu12 with crude cell wall that was pre-incubated with OsEGL1. Lane 3, reaction without Os4BGlu12 (−); lanes 4–6, reactions incubated with Os4BGlu12 (+) for 10, 30 and 60 min, respectively. (B) Os1 product incubated with Os4BGlu12. Lane 3, reaction without Os4BGlu12 (−); lanes 4–6, Os1 reaction incubated with Os4BGlu12 (+) for 10, 30 and 60 min, respectively. Lane 1 in (A) and (B), glucose (G) and laminarioligosaccharide standards of DP 2–7 (L2–L7); lane 2 in (A) and (B), cellooligosaccharide standards of DP 2–4 (C2–C4). Fifty microliters of reaction mixtures composed of Os4BGlu12 (1 μg) and cell wall hydrolysis products in 50 mM sodium acetate buffer, pH 5.0, were incubated at 37 °C and products analyzed by silica gel TLC with acetonitrile/water (3:1 by volume).

that Os1 and Os2 are (1,3;1,4)-linked glucotetraose ($G4G4G3G_{red}$) and glucotriose ($G4G3G_{red}$), respectively, while Os3 is the (1,3)-linked disaccharide of glucose ($G3G_{red}$) [where G represents a β-glucosyl residue, 3 and 4 are (1,3) and (1,4) linkages, respectively; and "red" indicates the reducing terminus].

3.5. Kinetic analysis

The substrate specificity of Os4BGlu12 toward various kinds of disaccharides and oligosaccharides of glucose was evaluated by kinetic analysis. For disaccharides, Os4BGlu12 hydrolyzed sophorose (β-1,2), laminaribiose (β-1,3), and cellobiose (β-1,4), but not gentiobiose (β-1,6). It hydrolyzed cellooligosaccharides with DP of 3–6 and (1,3;1,4)-β-glucooligosaccharides with DP of 3–4, but not laminarioligosaccharides with DP > 2. As shown in Table 1, Os4BGlu12 hydrolyzed laminaribiose with the highest catalytic efficiency (k_{cat}/K_m) value with a relatively high K_m and apparent k_{cat} values, but hydrolyzed sophorose and cellobiose with low efficiency. For cellooligosaccharides, there was a large decrease in K_m and increase in k_{cat} between DP of 2 and 3, after which the K_m remained approximately constant with increasing chain length of the substrate, while the apparent k_{cat} values increased slightly from DP of 3 to 5, but decreased at DP of 6. Catalytic efficiency (apparent k_{cat}/K_m) was within error for DP of 3 and 4, but increased 65% from DP of 4 to 5, so that the apparent k_{cat}/K_m of cellopentaose was 67-fold higher than that of cellobiose. An estimate of subsite affinities based on the assumptions of Hiromi et al. [24], gave apparent subsite affinities of 9.3 ± 0.4 kJ/mol for subsite +2, 0.3 ± 0.4 kJ/mol for the +3 subsite, 1.3 ± 0.2 kJ/mol for the +4 subsite and -0.7 ± 0.2 kJ/mol for the +5 subsite. Os4BGlu12 also hydrolyzed (1,3;1,4)-glucotriose with catalytic efficiency 15-fold lower than that of cellotriose and hydrolyzed (1,3;1,4)-glucotetraose with catalytic efficiency 4-fold lower than that of cellotetraose. The K_m values for all oligosaccharides tested, except for cellobiose, were nearly equivalent.

Os4BGlu12 exhibited broad glycone specificity against *p*NP glycosides, although it did not hydrolyze *p*NP β-D-mannoside, *p*NP β-D-cellobioside, *p*NP α-D-glucoside, *p*NP β-L-fucoside and *p*NP α-L-fucoside (Table 1). The enzyme hydrolyzed *p*NP glycosides with much lower K_m and higher apparent k_{cat}/K_m values than oligosaccharides. The enzyme hydrolyzed *p*NP β-D-fucoside with the highest k_{cat}/K_m value of 47 s^{-1} mM^{-1}. It hydrolyzed *p*NP β-D-glucoside, *p*NP β-D-xyloside and *p*NP α-L-arabinoside with similar catalytic efficiency (apparent k_{cat}/K_m of 23–25 s^{-1} mM^{-1}), while *p*NP β-D-galactoside was hydrolyzed with 35% of the efficiency of *p*NP β-D-glucoside.

Several available natural glycosides could be hydrolyzed at different rates (Table 1). Deoxycorticosterone 21-glucoside, a steroid glucoside from the adrenal cortex of vertebrates, was the most efficiently hydrolyzed glycoside with a high apparent k_{cat} value (13 s^{-1}) and low K_m value (0.68 mM). Apigenin 7-*O*-β-D-glucoside, a flavone glucoside, was hydrolyzed with low apparent k_{cat} (0.83 s^{-1}) and K_m (0.12 mM) values. Those that were hydrolyzed with relatively low catalytic efficiency (below 0.67 s^{-1} mM^{-1}) included indoxyl β-D-glucoside, D-amygdalin, and linamarin. The enzyme could not hydrolyze salicin, naringin (a 7-*O*-2-α-rhamnosyl-β-glucoside), gossypin (a flavonoid 8-*O*-glucoside), quercetin 3-β-D-glucoside (a flavonoid 3-*O*-β-glucoside) and phlorizin (phloretin 2′-*O*-β-glucoside, which is structurally similar to a flavonoid 5-*O*-β-glucoside).

4. Discussion

The high abundance of *Os4bglu12* transcripts in rice seedling shoots is similar to the rice *bglu1* and *bglu2* β-glucosidase genes [14]. However, differences were seen in 6-week-old mature rice plants in which *Os4bglu12* gene was highly expressed in leaf sheaths and stems, while *bglu1* is highly expressed in flowers and *bglu2* in the node. The high expression of *Os4bglu12*, *bglu1* and *bglu2* in different tissues of mature rice plants might indicate functional differences between different β-glucosidase isozymes, although they may play similar roles in different tissues.

The expression of *Os4bglu12* was up-regulated in response to wounding, methyl jasmonate and ethephon treatments, with high mRNA levels observed within 10 h after treatment. Consistent with this study, Wang et al. [18] reported that the transcript level of EST contig BHPiw028, corresponding to *Os4bglu12*, increased in response to brown planthopper attack, based on subtractive hybridization cDNA library screening. Methyl jasmonate and ethephon have been reported to be involved in wound signaling in several plant species [25–27]. This implies that induction of *Os4bglu12* gene expression by wounding and herbivore attack may be mediated by the methyl jasmonate and ethylene signal transduction pathway. The protein product of *Os4bglu12* gene may be involved in cell wall recycling or release of active compounds from glycosides for defense. In addition, the levels of transcripts that hybridized to the ESTs BE607353 and BG101702, which correspond to the *Os3bglu7* and *Os4bglu12* β-glucosidase genes, respectively, were induced by salt stress in salt-tolerant rice (var Pokkali), but not in the salt-sensitive cultivar IR29 [17]. These studies indicated that Os4BGlu12 may have functional roles in response to stresses.

One possible role of Os4BGlu12 is hydrolysis of cell wall-derived oligosaccharides. Os4BGlu12 efficiently hydrolyzed cellooligosaccharides (β-1,4-linked) with DP of 3–6 and laminaribiose (β-1,3), which is more like Os3BGlu7, Os3BGlu8, and Os7BGlu26 than Os3BGlu6, which hydrolyzes laminaribiose, but not cellooligosaccharides [28,15,16]. The large increase in catalytic efficiency of Os4BGlu12 with increasing chain length from DP of 2 to 3 is similar to rice Os3BGlu7 [28], rice Os3BGlu8 and Os7BGlu26 β-glucosidases [15] and different from barley β-glucosidase βII, which hydrolyzed cellobiose better than cellotriose [29]. However, the K_m values of barley β-glucosidase βII and rice Os3BGlu7, Os3BGlu8 and Os7BGlu26 β-glucosidases decreased with increasing DP from 3 to 6, while Os4BGlu12 was approximately constant, so Os4BGlu12 showed a much lower dependence on chain length over this range. The preference of the rice Os4BGlu12 for (1,4)-β-oligosaccharides suggested that it has an extensive subsite binding region, but primarily binds the third glucosyl residue of cellooligosaccharides at subsite +2 with a weaker interaction at the 5th residue at subsite +4.

Akiyama et al. [19] reported the transcript level of the rice *OsEGL1* gene, which specifically hydrolyzes (1,3;1,4)-β-glucans, increased in rice seedlings in response to wounding, methyl jasmonate and ethephon, which is similar to the expression of *Os4bglu12*. Os4BGlu12 could hydrolyze (1,3;1,4)-glucooligosaccharides released from rice cell wall by OsEGL1 with catalytic efficiency that increased 4-fold with increasing chain length from 3 to 4, in contrast to cellotriose and cellotetraose, which are hydrolyzed more efficiently than (1,3;1,4)-oligosaccharides, but with no significant increase in efficiency between DP of 3 and 4. This result implies that enzyme may bind different linkages in different modes, so that it can play a role in cellooligosaccharide hydrolysis and may cooperate with OsEGL1 in hydrolysis of (1,3;1,4)-β-glucans of cell walls.

The above studies suggest roles for Os4BGlu12 in release of glucose from oligosaccharides to complete the depolymerization of at least three major cell wall polysaccharides present in rice tissues, including the (β-1,4); (β-1,3) and β-(1,3;1,4)-glucans. The reorganization and alteration of these cell wall β-glucans may be required during normal developmental processes or in response to environmental stresses. There have been reports that the hydrolysis of cell

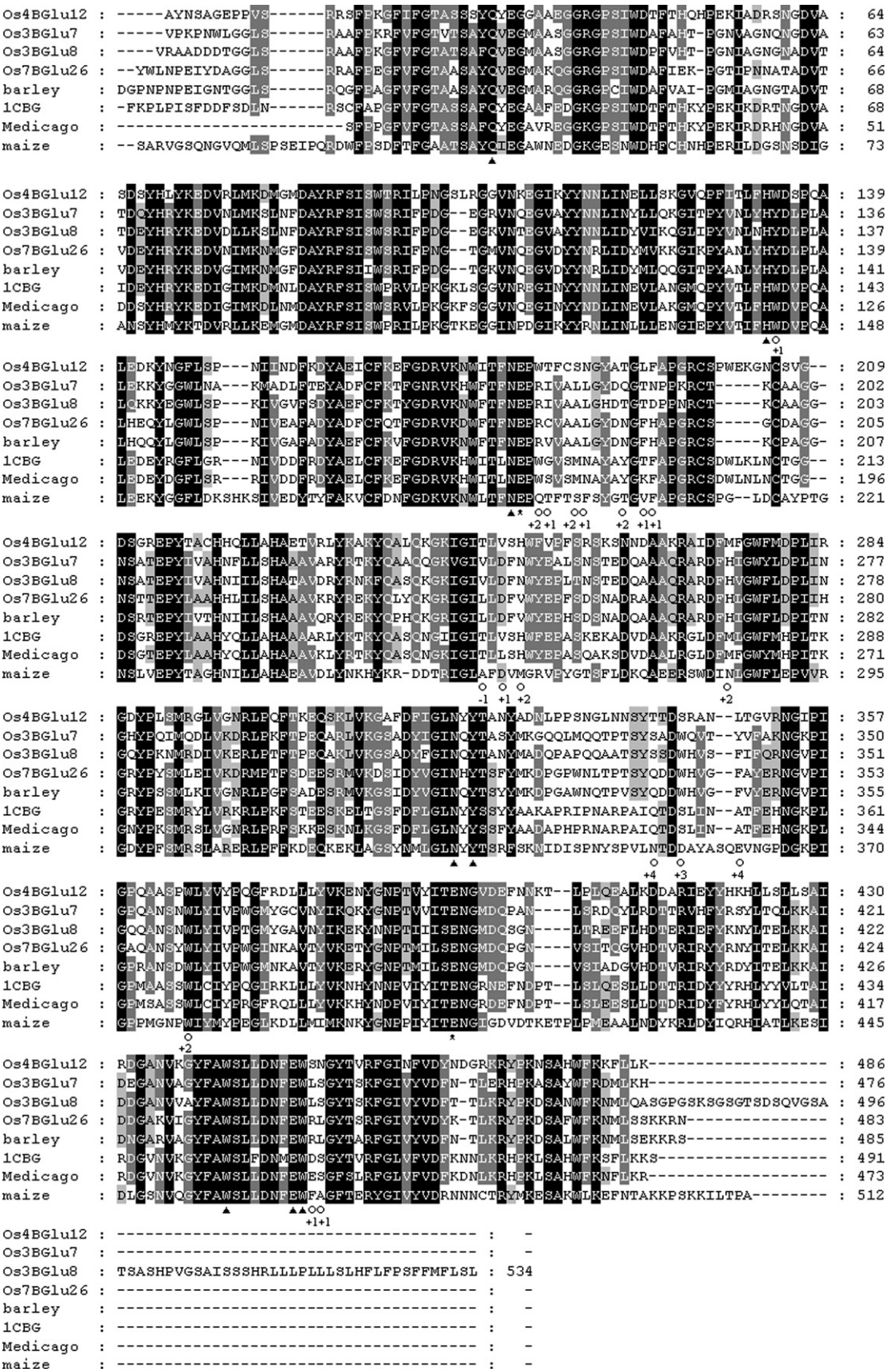

Fig. 4. Amino acid sequence alignment of rice Os4BGlu12 with related β-glucosidases. The rice cDNA derived sequences are labeled as Os4BGlu12, Os3BGlu7 (GenBank ID U28047), Os3BGlu8 (GenBank ID AK120790), and Os7BGlu26 (GenBank ID EU835514). Maize is maize β-glucosidase 1 (GenBank ID U33816); barley is barley βII β-glucosidase (GenBank ID ACF07998); 1CBG is white clover cyanogenic β-glucosidase (GenBank ID ABV54745); Medicago is *Medicago truncatula* isoflavone β-glucosidase (GenBank ID ABW76288) [31]. Stars represent the catalytic acid/base and nucleophile residues. Open circles with the number beneath represent residues to be at the −1, +1, +2, +3 and +4 subsites based on the Os3BGlu7 3D structure (PDB ID 1RGM and 3F5K) as predicted by Chuenchor et al. [35] and Kuntothom et al. [15]. Triangles mark the amino acids in close contact with the glucose residue at −1 subsite in Os3BGlu7 [35]. The alignment was generated using the Clustal X implementation of Clustal W [38,39].

wall polysaccharides also likely generates sugar signals [30]. Therefore, a role for Os4BGlu12 in degradation or modification of such signals cannot be ruled out.

Os4BGlu12 could hydrolyze deoxycorticosterone 21-glucoside and apigenin 7-*O*-β-D-glucoside with high catalytic efficiency, suggesting it could also play a role in releasing active steroids or flavonoids from their glycosides for defense. The fact the enzyme hydrolyzed apigenin 7-*O*-β-D-glucoside, but not flavonoid 3-*O*-glucoside and 8-*O*-glucoside, suggests it may have preference for the 7-*O*-linkage in flavonoid glucosides. Recently, Naoumkina et al. [31] reported that methyl jasmonate induced the transcript levels of four flavone/isoflavone β-glucosidases in cell suspensions of the legume *Medicago truncatula* within 30 min to 2 h, and the accumulation of the free isoflavonoid phytoalexin medicarpin released from its glycosides. Although Os4BGlu12 is thought to function in hydrolysis of oligosaccharides released from the cell walls, it is possible that this enzyme plays more than one role. Recently barley β-glucosidase βII, which is thought to help in hydrolysis of cell wall oligosaccharides during germination, has been found to hydrolyze cyanogenic glycosides from barley leaves [32]. This gives support to the possibility of one enzyme playing roles in both cell wall hydrolysis and defense.

The preference of the glycone moieties of Os4BGlu12 is different from the GH1 rice enzymes in that it could not hydrolyze *p*NP β-D-mannoside, but it instead hydrolyzed *p*NP β-D-xyloside and α-L-arabinoside with k_{cat}/K_m values equivalent to that for *p*NP β-D-glucoside [28,15,16]. The efficient hydrolysis of β-D-xyloside, is similar to white clover β-glucosidase, but is not generally found in GH1 enzymes that have been characterized [33]. The high activity upon *p*NP β-D-xyloside might result from a different positioning of subsite −1 residues, especially E449 and Q29 which correspond to residues that interact with O-6 of sugar residue, E451 and Q39, in *Spodoptera frugiperda* β-glycosidase [34]. This equivalent glutamate residue (Glu464) in maize ZmGlu1 β-glucosidase also specifically binds to O-6 and displayed a different conformational state in the crystal structure of ZmBGlu1 bound to the transition-state mimic glucotetrazole when compared the apo enzyme of ZmGlu1 [12]. Verdoucq et al. [12], stated that this conformational change might guide the movement of the glucosyl moiety into the correct distorted position required for nucleophilic attack to occur. In the case of rice Os3BGlu6 β-glucosidase, two conformations were observed for the corresponding glutamate (Glu451) in the apo enzyme structure, while it was locked in a single conformation in its covalent complex with 2-deoxy-2-fluoroglucoside [16]. The entropy loss upon glucoside binding was used to explain the high relative activity of Os3BGlu6 toward *p*NP β-D-fucoside. However, Os3BGlu6 has low activity toward *p*NP-β-D-xyloside, so the presence of carbon 6 of the pyranose ring must be more critical for Os3BGlu6 than for Os4BGlu12.

Since the substrate specificity of Os4BGlu12 is distinct from other rice enzymes, a protein sequence alignment was performed to compare the amino acids lining the binding site of Os4BGlu12 with the residues found at the −1, +1, +2, +3 and +4 subsites in the Os3BGlu7 X-ray crystal structure [35] and PDB ID 3F5K (Fig. 4). All the residues in close contact with the sugar at the −1 subsite were conserved in all enzymes, except for V241, which is in contact with E176 catalytic acid/base in Os3BGlu7. Therefore, differences in glycone specificities might derive from a different positioning of subsite −1 residues [12,36]. Rice Os3BGlu7, Os3BGlu8, Os7BGlu26 and barley βII, were classified in a distantly related group and shared 51–53% amino acid sequence identity with Os4BGlu12 [13]. They possess almost completely different amino acids at the residues predicted to form the +1 to +4 subsites compared to Os4BGlu12. However, residue W358 at subsites +1 and +2 in Os3BGlu7, which is a conserved Trp shown to position the aglycone of other plant GH1 β-glucosidases, was conserved in all enzymes [11,12]. Os4BGlu12 is more closely related to the dicot defense enzymes white clover cyanogenic β-glucosidase [37] and *M. truncatula* isoflavone β-glucosidase [31], with which it shares 63 and 65% protein sequence identity, respectively, and these enzymes have many identical predicted active site amino acid residues. The above-mentioned differences and similarities in active site amino acid residues between Os4BGlu12 and the other enzymes may result in the unique overall binding site geometry that allows Os4BGlu12 to hydrolyze glucooligosaccharides with different linkages, *p*NP glycosides with a range of glycones and flavonoid and steroid glucosides. However, the overall sequence difference or similarity of Os4BGlu12 with other enzymes may not reflect the aglycone specificity of the enzyme, and the molecular basis of the specificity based on the interactions with the aglycone remains to be studied.

5. Conclusions

In summary, this work explored the possible functions of rice Os4BGlu12, the transcript level of which was previously found to be induced in response to herbivore attack and salinity stress [17,18]. The expression of this gene was also up-regulated in response to wounding and wound-signaling related phytohormones. Under normal growth conditions, high transcript levels were detected in the shoot of rice seedlings and leaf sheaths and stems of 6-week-old mature rice plants. The recombinant Os4BGlu12 hydrolyzed three types of substrates, including oligosaccharides, a steroid glycoside, and a flavonol glycoside, which suggests that it might play multiple roles in rice, such as in defense and cell wall remodeling during development or wounding. The similar expression patterns of rice OsEGL1 endo-(1,3;1,4)-β-glucanase and Os4BGlu12 β-glucosidase in wounding response and the action of Os4BGlu12 on the products of OsEGL1 implies that they may act in a concerted fashion. Furthermore, the amino acid residues predicted to line the active site of Os4BGlu12 are quite distinct from those in other oligosaccharide hydrolases, but similar to those of cyanogenic and flavonoid β-glucosidases, consistent with an additional possible role in hydrolysis of defensive glycosides. However, an additional defensive role remains to be confirmed with natural substrates isolated from rice.

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